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PRINCIPAL INVESTIGATOR: Steffi Oesterreich, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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6. AUTHOR(S)

Steffi Oesterreich, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

E-Mail: steffio@breastcenter.tmc.edu

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Loss of G2/M checkpoint plays an important role in tumorigenesis, however, few genes involved in this checkpoint control have been shown to be deregulated in human breast tumors. SAFB1 is a multifunctional protein which maps to a locus of high LOH, and mutations have been identified from both breast cancer cell lines and tumors. Our preliminary data show that inactivation of SAFB1 in MEFs result in loss of G2/M checkpoint control, and that loss of SAFB1 expression is associated with Taxotere resistance in human breast tumors.

We therefore hypothesize that SAFB1 is critical for G2/M checkpoint control, and that its inactivation results in resistance to breast cancer therapies that utilize a block in G2/M and subsequent apoptosis. We will identify the mechanism by which SAFB1 controls the G2/M checkpoint, and will subsequently analyze whether Taxotere-resistant tumors show altered expression of genes involved in these pathway(s).

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Introduction:

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checkpoint, and will subsequently analyze whether Taxotere-resistant tumors show altered expression of genes involved in these pathway(s).

Body:

We have made progress in all three aims as outlined below.

Aim 1) Is the G2/M checkpoint lost in mammary epithelial cells from the SAFB1$^{-/-}$ knockout mice? Our preliminary data from MEFs suggest that SAFB1 is involved in G2/M checkpoint control. To confirm and extend these preliminary studies in a more relevant system, we will:

1.1) Grow primary mammary epithelial cells from $^{+/+}$ and $^{-/-}$ mice in culture, and expose them to γ-radiation and to agents including chemotherapeutic drugs causing mitotic defects (Taxotere, Nocodazole, Colcemid), and analyze G2/M block (FACS and apoptosis analysis).

1.2) Investigate whether SAFB1 loss leads to polyploidy and chromosome missegregation.

Progress:

We have begun to grow primary mammary epithelial cells (MEC) in culture, and have included growing them in three dimensions (3D), rather than only on plastic (Schmeichel *et al.* 2003). There is increasing and overwhelming evidence that only the 3D culture faithfully reconstitute the normal functions of cells in culture, and we thus would like to utilized this model for our assays. We entered a collaboration with Dr. Dan Medina (Baylor College of Medicine [BCM], Houston), who is experienced in growing MECs. We have made significant progress in mastering this challenging technique, and I expect that we will be able to perform the actual experiments within the next 6 months.

We have tested polyploidy by FACS analysis, and failed to detect any significant difference between wiltype and knockout MEFs. However, we have complemented these studies with comparative genomic hybridization (CGH) studies (collaboration with Dr. Rao Pulivarthi, BCM), and have made the exciting observation that the SAFB1 ko MEFs show amplification of the X-chromosome. These data are preliminary, and need to be confirmed by FISH analysis, which we plan to do in the next 3 months.

Aim 2) What is the mechanism for the SAFB1-mediated G2/M checkpoint control? Because our preliminary data are indicative of defective spindle checkpoints in SAFB1$^{-/-}$ MEFs, we will:

2.1) Analyze the two closely linked components of the mitotic spindle, the centromere-kinetochore and the kinetochore-microtubule attachment, in SAFB1$^{+/+}$ and SAFB1$^{-/-}$ cells by immunofluorescence.

2.2) Determine which SAFB1 domains are necessary and sufficient for G2/M arrest. Therefore we will compare how reintroduction of wildtype SAFB1 and SAFB1 mutants lacking DNA-binding and protein-protein interaction domains restores the G2/M checkpoint in SAFB1$^{-/-}$ cells.

2.3) Identify SAFB1-interacting proteins which participate in the same spindle checkpoint pathways through immunoprecipitation and subsequent sequencing of proteins which specifically interact with SAFB1 in mitosis.

Progress:

We have recently made the exciting observation that the SAFB1-mediated resistence to docetaxel is increased in the presence of c-myc; SAFB1 ko MEFs overexpressing ras/myc but not ras/SV40T show increased resistance to the effects of docetaxel. We also observed that the SAFB1 ko MEFs spontaneously immortalize which was associated with a loss of the tumor suppressor and cell cycle inhibitor p19ARF (Wadhwa *et al.* 2004). Since p19ARF has been shown to be critical for myc-induced apoptosis (Cleveland *et al.* 2004, Qi *et al.* 2004, Zindy *et al.* 1998), the possibility arises that loss of SAFB1 results in docetaxel resistance due to loss of myc-induced apoptosis. Thus, these new data suggest that SAFB1 might be induced in drug resistance by loss of apoptosis. If and how this is connected to our observation of loss of G2/M checkpoint needs to be determined. Currently, we are testing other drugs commonly used in chemotherapeutic treatment to see whether the resistance is specific for docetaxel. These studies will help us to decipher G2/M-specific and/or apoptosis-specific events.

We have also finished making different constructs to perform the experiments proposed in Aim 2.2. We have successfully cloned ?RD (repression domain), ?SAF-Box (scaffold attachment factor Box – DNA binding), and ? RRM (RNA recognition motif) into expression vectors. We are now planning on reintroducing full length, or the different deletion mutants into the KO cells which will also help us in understanding the mechanism by which SAFB1 refers resistance.

We have not yet begun to analyze interaction of SAFB1 with mitotic proteins, but expect to start these studies in Year 2.

The data describing the immortalization and docetaxel resistance in SAFB1 KO MEFs, together with earlier observations of loss of SAFB1 and worse clinical outcome, have been written up, and will be submitted to Cancer Cell within the next few days.

3) Are genes which are mechanistically linked to SAFB1-mediated checkpoint control also deregulated in Taxotere-resistant breast cancer?

3.1) Analyze whether inactivation of candidate genes (identified in Aim 2.3) also leads to Taxotere resistance in cell line models.

3.2) Analyze expression of these genes in Taxotere resistant breast tumors.

Progress:

Due to our recent identification of myc in SAFB1/p19ARF-mediated cell response to docetaxel, we have reanalyzed Dr. Chang's microarray data (Chang *et al.* 2003), and found that myc was overexpressed, along with loss of SAFB1, in resistant tumors, confirming our data from the MEFs.

We have not started any confirmatory studies with the patient material yet, but expect to do this within the next 3 months.

Key Research Accomplishments:

1) Amplification of x-chromosome in SAFB1 Ko MEFs as determined by CGH
2) Increased resistance to Taxotere in SAFB1 ko cells in the presence of myc
3) Spontaneous immortalization of SAFB1 KO MEFs, associated with loss of $p19^{ARF}$, suggesting that loss of p19ARF could contribute to decreased apoptosis
4) Inverse correlation of SAFB1 and myc in breast cancer samples (analysis of previously performed microarray studies); loss of SAFB1 and increase of myc was significantly associated with resistance
5) Successful generation of SAFB1 deletion constructs to be used to decipher mechanisms

Reportable Outcomes

Poster Presentation:

Dobrzycka KM*, Kang K, Ivanova M, Jiang S, Rao P, Lee AV, Oesterreich S. SAFB1 as a tumor suppressor: its role in immortalization, transformation, and genomic instability. 27^{th} Annual San Antonio Breast Cancer Symposium. San Antonio, TX. December, 2004.

Dobrzycka KM, Oesterreich S. The Scaffold Attachment Factor SAFB1: A new player in G2/M checkpoint control? DOD Era of Hope June 2005, Philadelphia.

Oral Presentation:

Dobrzycka KM*, Oesterreich S. SAFB1 As a Novel Tumor Suppressor Gene in Breast Cancer. Gordon Research Conference: Cancers Models and Mechanisms. Newtown, RI. August 2004. (Oral presentation)

Manuscripts:

Klaudia M. Dobrzycka, Kaiyan Kang, Shiming Jiang, Rene Meyer, Rao Pulivarthi,Valerie Bardou, Anna Tsimelzon, Jenny Chang, C. Kent Osborne, Adrian V. Lee, and Steffi Oesterreich. Disruption of Scaffold Attachment Factor SAFB1 Blocks $p19^{ARF}$ Induction, Immortalizes Cells, and Worsens Breast Cancer Prognosis and Docetaxel Resistance. To be submitted to Cancer Cell.

Conclusions:

We have confirmed that loss of SAFB1 results in resistance to Taxotere (docetaxel), and have recently determined that this resistance can especially be observed in cells which also overexpress myc. We have also made the exciting observation that SAFB1 null cells spontaneously immortalize which was associated with loss of p19ARF. Together with our previous results, these data suggest that loss of SAFB1 might result in loss of apoptosis, since p19ARF is a critical mediator of myc-induced apoptosis.

We are currently trying to decipher whether the resistance (and the loss of apoptosis) is specific for G2/M drugs, or whether it can also be seen in the presence of other commonly used chemotherapeutic drugs. During the next year we will use various SAFB1 mutants which should provide critical information regarding the mechanism. We will also use tumor material from the neoadjuvant clinical study (collaboration with Dr. Chang) to measure expression of SAFB1, myc, and other genes which we hope to identify as critical players in the SAFB1-mediated resistance phenomena.

Collectively, our data point to a critical role of SAFB1 as a breast tumor suppressor gene.

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Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, Mohsin S, Osborne CK, Chamness GC, Allred DC, *et al.* 2003 Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* **362** 362-9

Cleveland JL & Sherr CJ 2004 Antagonism of Myc functions by Arf. *Cancer Cell* **6** 309-11.

Qi Y, Gregory MA, Li Z, Brousal JP, West K & Hann SR 2004 p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* **431** 712-7. Epub 2004 Sep 8.

Schmeichel KL & Bissell MJ 2003 Modeling tissue-specific signaling and organ function in three dimensions. *Journal of Cell Science* **116** 2377-88

Wadhwa R, Sugihara T, Taira K & Kaul SC 2004 The ARF-p53 senescence pathway in mouse and human cells. *Histology and Histopathology* **19** 311-6.

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ & Roussel MF 1998 Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes and Development* **12** 2424-33.